DE-FG02-88ER13862

GENETICS OF BACTERIA THAT OXIDIZE ON-CARBON COMPOUNDS
PRINCIPAL INVESTIGATOR: R.S. HANSON

I. Reprints of manuscripts

1. Xu, H.H., J.J. Janka, M. Viebahn and R.S. Hanson. 1995. Nucleotide sequence of the *mxc*Q and mxcE genes, required for methanol dehydrogenase synthesis in *Methylobacterium organophilum* XX: a two component regulatory system. Microbiology **141:** 2543-2551.

2. Hanson, R.S. and T.E. Hanson. 1996. Methanotrophic Bacteria. Microbiological Reviews. **60:** 439-471.

II. Progress on Objectives.

Objective 1: Further define the Regulation of methanol dehydrogenase (MDH) synthesis in *M. organophilum*.

Most of our efforts have been directed toward this objective because genetic and sequence information indicated that a novel regulatory system more complicated than other two component, positively acting, regulatory systems identified in procaryotes is involved in the expression of MDH. The focus of the new proposal is limited to studies of the regulation of MDH synthesis.

1a. Identify regulatory protein binding sequences

A 170 bp oligonucleotide sequence is found between the transcriptional start and translational start sites preceding the *mxa*F open reading frame that encodes the large subunit of MDH. It was considered possible that this may have played a role in transcriptional attenuation or activation. However, it has been shown that deletion of this sequence and fusing the mxaF promoter to a promoterless *xyl*E gene did not affect production of catechol 2,3-dioxygenase. Expression of XylE still was induced by methanol and required a functional *mxa*B, *mxb*D,N,M and *mxc*Q,E genes. The total activity of catechol 2,3-dioxygenase was similar with and without this sequence downstream of the *mxa*F promoter when fused to the *xyl*E gene. The sequences required for maximum inducible expression of *mxa*F have been identified by deletion analysis as those between positions 63 and 220 of Figure 1. Deletions extending to position 104 reduce expression of mxaFp::xylE promoter fusions by about 50%. Deletions into the first region of diad symmetry (positions 113-145) completely abolish expression of *xyl*E in promoter fusions (Xu *et al*, 1995). The data for these conclusions is presented in Xu *et al* (1995).

We have also fused the mxaW promoter (see Figure 1) to *xyl*E (Xu *et al*, 1995) using an inversion of the mxaF promoter and have subsequently shown that deletion of nucleotides within the inverted repeat (positions 156-176, Figure 1) cause loss of catechol 2,3 dioxygenase activity when induced by methanol. Catechol 2,3 dioxygenase activity with the *mxa*Wp::*xyl*E fusion is only about 15% of the acitivity as with the *mxa*Fp::*xyl*E promoter fusions{ ADDIN ENRef }. These data suggest that the putative positive regulator binding site postions (156-176) that have been shown by footprinting experiments to be the site of DNA protein binding and the other upstream region of diad

DISCLAIMER

symmetry are important for transcription in both directions (H. Xu, Ph.D. thesis, Univeristy of Minnesota).

At this time, it has been shown that the purified MxcE protein and the GST-MxbM fusion protein have been shown to bind to a 219 bp oligonucleotide sequences upstream of *mxa*F in gel retardation assays. The fusion proteins GST-MxcE and GST-MxbM (see below) as well as the purified MxcE protein protein bind to the same oligonucleotides. In our initial experiments, the MxbM protein cleaved from the fusion protein does not bind in all assays. This may be due to problems with inactivation during cleavage or to carryover of Triton X-100. Autophosphorylation of purified MxcE in the presence of acetyl-phosphate and magnesium improves its binding to the oligonucleotide. The retarded bands resulting from binding of MxcE, GST-MxbM or a combination of the two migrate more rapidly than retarded bands produced by incubating crude extracts with the 219 bp oligonucleotide. This may be the result of the binding of more than these two proteins to the oligonucleotide when crude extracts are used.

1b. Sequence genes required for expression of methanol dehydrogenase and characterize the gene products.

We have previously shown that two unlinked groups of genes (*mxb*M,N,D) and *mxc*Q and E) as well as a gene found downstrean of *mxa*F (*mxa*B) are all required for the expression of *mxa*F in *M. organophilum* XX { ADDIN ENRef }. During this grant period, we have sequenced all of these genes and have shown that the deduced amino acid sequences of MxcQ and MxbM are similar to different groups of sensor-histidine-kinases that are components of two component regulatory proteins while MxaB and MxcE belong to one group of response regulators including MxaX of *Paracoccus denitrificans* { ADDIN ENRef }, DegU of *B. subtilis* { ADDIN ENRef } NarL of *E. coli* { ADDIN ENRef } and UhpA of *S. typhimurium* { ADDIN ENRef }. MxcE and MxaB have 65.6% similarity (65.6% identity) while MxaB and MxcE both have 45% similarity to MxbM. MxbM belongs to a subfamily of response regulators that differ significantly from the subfamily containing MxaB and MxcE.

We have cloned the *mxc*E and *mxb*M genes into pGEX-2TK by fusion to the glutathione S-transferase gene. After transformation into *E. coli* JM109, the plasmid was isolated and sequenced to be sure the reading frame was proper and the recombinant plasmid was transferred to *E. coli* BL21 (DE3). The proteins were expressed and the fusion proteins were purified using the glutathione Sepharose 4B purification procedure of Pharmacia.

Antibodies were prepared against the GST-MxcE fusion protein in rabbits and were absorbed with *E. coli* BL21 (DE3) (pGEX-2TK). The antibodies were used in Western blots to detect MxcE in cell extracts and to determine if it is produced in both induced and noninduced conditions. We have shown this protein to be present cells grown on a mineral salts medium with methanol as well as Penassay broth.

Objective 1c. Sequence the *mox*W gene. This work was delayed in order to complete sequencing of the gene clusters containing the *mxa*B and *mxb*D,N,M genes. We wish to know if other genes involved in expression of MDH may be located in this region. At this time we have not found sequence similarity to the partial sequence of *mxa*W and other genes or proteins in the data bases searched.

Objectivwe 2: Identification of genes and proteins involved in copper expression of sMMO in *M. trichosporium OB3b.*

Our initial attempts to complement mutants lacking sMMO activity with partial *Sau* 3A fragments cloned into a pLA2917 vector { ADDIN ENRef } were unsuccessful due to instability of all of the sMMO mutants available and the slow growth of recombinants.

Objective 3: Clone and attempt to express sMMO genes from M. trichosporium in *M. organophilum* XX.

In response to comments received after review of the previous proposal we did not attempt to clone sMMO in this bacterium. However, we have collaborated with Dr T. Wood's laboratory expression of sMMO in *Rhizobium meliloti* , *Agrobacterium tumefaciens*, and *Pseudomonas putida*. has been demonstrated. (D. Jahng, C.S. Kim, R.S. Hanson, and T.K. Wood. 1996. Optimization of trichloroethylene degradation using soluble methane monooxygenase of *Methylosinus trichosporium* OB3b expressed in recombinant bacteria. Biotechnology and Bioengineering **5** 1:349-359).

Objective 4: Further experiments to determine if *M. organophilum* is capable of growth as a facultative methylotroph.

We have previously demonstrated that restriction fragments from methane and methanol grown cells are indistinguishable and will use fluorescently labelled oligonucleotides that are complementary to 16S rRNA sequences present in the large subunit rRNAs of are *M. oarganophilum* XX in order to demonstrate that all cells in the culture are *M. oarganophilum* XX and not another methanotroph. The techniques have been described in a previously published manuscript { ADDIN ENRef }

Figure 1. Sequences upstream of *mxa*F. Shine-Delgarno (S-D) sequences and -10, -35 promoter sequences for *mxa*F and *mxa*W are identified. Transcriptional and translational start sites for each gene are identified with the arrows indicating the directions in which translation and transcription occur.

```
1                                          AvaI                                    80
AGGCCATGCC AGCCTCGCGC CATCACTGCT CCTCCCGATG TCTCGGGCCG CTGCCGCCCG TTCCGCCTAG TGGTGCGCCG
TCCGGTACGG TCGGAGCGCG GTAGTGACGA GGAGGGCTAC AGAGCCCGGC GACGGCGGGC AAGGCGGATC ACCACGCGGC
      <------------------|      S-D mxaF                <---------|       -10W
          moxW ORF                                 mxaW transcriptional start


                                                                                   160
GGCCTTCGTC GCGGTCAAGC CGGAGGGATA CGAGAAGATC GCCAATTTAG AAATGCCACA TCTTCCCAGC AAGAAATGTC
CCGGAAGCAG CGCCAGTTCG GCCTCCCTAT GCTCTTCTAG CGGTTAAATC TTTACGGTGT AGAAGGGTCG TTCTTTACAG
             -35W                    >>>>>>      >>>>   <<<<     < <<<<<           <<<<


           -35F                          -10F      |mxaF transcriptional start ------>     240
TGACCGGGTA AAGACATCTC CTTCAATCAA CGCCTAGAAA CGATAGGCGA CGGGTGCTAA TAAGTCTTTC ATAAGACAGA
ACTGGCCCAT TTCTGTAGAG GAAGTTAGTT GCGGATCTTT GCTATCCGCT GCCCACGATT ATTCAGAAAG TATTCTGTCT
<            >>>>>>


                                                                                   320
GTTTATCTAA GATTGTTCTT AGATTTTCTG CTCGGTCGAG GAATGGACTT CGTGGCGAAC CCTGAACCTC AGAGGGAATA
CAAATAGATT CTAACAAGAA TCTAAAAGAC GAGCCAGCTC CTTACCTGAA GCACCGCTTG GGACTTGGAG TCTCCCTTAT


          KpnI                                  S-D mxaF       |mxaF ORF--->
GCTCAAGAGG TACCGCGAGG CCAAAGGCGT GGCGGTCAAG CGAGGAGACG CAGGATGA
CGAGTTCTCC ATGGCGCTCC GGTTTCCGCA CCGCCAGTTC GCTCCTCTGC GTCCTACT
```